## REMARKS/ARGUMENTS

**Claim rejections under 35 USC § 103**

*Claims 1-2, 4-6, 12-13, 25-26, 28-30, 36-37, 49, and 53-55 are rejected under 35 U.S.C. 103(a), as being unpatentable over Dunder E. et al. in Maize Transformation by Microprojectile Bombardment of Immature Embryos; Springer-Verlag, Berlin-Heidelberg; pages 127-138.*

Applicants traverse the rejection. In order to expedite prosecution Applicants have amended Claims 1, dependent claims thereof, and Claim 55. Claims 1 and 55 now indicate that the immature embryo will not come in contact with an external source of auxin before the bombardment step is implemented.

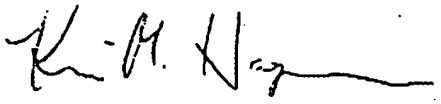
Applicants respectfully point out that within the Vain et al. (1993), on page 84 under materials and methods, it states that the medium used contains 1.5 mg/l of 2,4-D (Appendix A). This auxin-containing medium is used through out the protocol. On page 85, top of column 1, the osmotic treatment described is the initiation medium differing only in the concentration of sorbitol and/or mannitol concentrations. The Vain et al. (1993) publication is attached to this response. Dunder et al. (1995) states, on page 134, step 3, that prior to bombardment, "[p]late the explants, embryo axis down, on a medium capable of inducing somatic embryogenesis (as described earlier)." The publications do not teach stable transformation without the use of auxin prior to or during bombardment. The claims are therefore nonobvious.

Applicants again point out that the publications indicate that stably transformed maize plants were only obtained in experiments where the immature embryos were bombarded a day or more after isolation. Claims are limited to stable transformation.

## CONCLUDING REMARKS

Applicants have amended Claims 1, 4, 5, 6, and 55. Claims 2, 12, and 13 have been cancelled. Claims 1, 4-6, 25-26, 28-30, 36-37, 49, and 53-55 are now pending. No new matter has been added by amendment. Reexamination and reconsideration of the claims as amended are respectfully requested. In view of the above comments and amendments, withdrawal of the outstanding rejection and allowance of the remaining claims is respectfully requested.

Respectfully submitted.



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# Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize *

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Summary. The effects of osmotic conditioning on both transient expression and stable transformation were evaluated by introducing plasmid DNAs via particle bombardment into embryogenic suspension culture cells of *Zea mays* (A188 x B73). Placement of cells on an osmoticum-containing medium (0.2 M sorbitol and 0.2 M mannitol) 4 h prior to and 16 h after bombardment resulted in a statistically significant 2.7-fold increase in transient ß-glucuronidase expression. Under these conditions, an average of approximately 9,000 blue foci were obtained from 100 µl packed cell volume of bombarded embryogenic tissue. Osmotic conditioning of the target cells resulted in a 6.8-fold increase in recovery of stably transformed maize clones. Transformed fertile plants and progeny were obtained from several transformed cell lines. We believe the basis of osmotic enhancement of transient expression and stable transformation resulted from plasmolysis of the cells which may have reduced cell damage by preventing extrusion of the protoplasm from bombarded cells.

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid, PCV = packed cell volume, GUS = ß-glucuronidase, NOS = nopaline synthase, PIG = Particle Inflow Gun, PPT = phosphinothricin.

## Introduction

Particle bombardment is valuable for both gene expression (Ludwig *et al.* 1990) and stable transformation research (Christou *et al.* 1988). The basis of particle bombardment is the acceleration of small DNA-coated particles toward cells resulting in the penetration of the protoplasm by the particles and subsequent expression of the introduced DNA. With certain plants, particle bombardment is currently the most efficient method for introduction of foreign DNA. Although there have been many reports on optimization of physical bombardment parameters (Klein *et al.* 1988) and modification to the actual bombardment device (Williams *et al.* 1991; Sautter *et al.* 1991; Finer *et al.* 1992), limited data has been reported on cell preparation methods to make the target tissue more receptive to particle gun-mediated transformation.

Benefits from culture venting (Russell *et al.* 1992), cell filtration (Finer *et al.* 1992), and the use of cells in the proper phase of growth (Armalco *et al.* 1990) or at the proper density (Finer *et al.* 1992) have been reported for different species using the particle gun. Another factor affecting the efficiency of particle gun-mediated transformation is osmotic treatment of target tissues. A 7- to 10-fold enhancement in stable transformation of microorganisms (Armaleo et al. 1990; Shark *et al.* 1991) and nonembryogenic plant cells (Russell *et al.* 1992) was reported following culture on media containing mannitol and sorbitol.

Particle gun-mediated transformation of *Zea mays* has been reported by several laboratories (Fromm *et al.* 1990; Gordon-Kamm *et al.* 1990; Walters *et al.* 1992) and is currently the most efficient technique for production of fertile, transgenic maize plants. In this paper, we describe the effect of osmotic treatment on transient expression and stable transformation of embryogenic maize cells and the recovery of fertile transgenic maize plants.

## Materials and Methods

*Plant Tissue Preparation:* Type II embryogenic callus cultures of maize (*Zea mays* A188 x B73) were initiated and maintained on $AgNO_3$-containing medium as described previously (Vain *et al.* 1989). Embryogenic suspension cultures were initiated from type II embryogenic callus in a medium containing MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al.* 1968), 2% sucrose, and 1.5 mg/l 2,4-D (pH 5.7). The suspension cultures were maintained in 125 ml DeLong flasks by weekly subculture of 10-20 µl PCV of tissue into 30 ml of fresh medium. Cell culture at very low density was a determinant factor for rapid establishment and easy maintenance of homogeneous, fast-growing embryogenic suspension cultures. The suspension cultures were maintained in the light (30 $\mu Em^{-2}s^{-1}$; 16 h) at 150 rpm. Prior to bombardment, embryogenic maize cells were filtered through a 500 µm filter and 100 µl PCV was evenly dispersed on a 7 cm filter paper disc (Whatman #4) forming a very thin layer of cells. Discs were stored on the maintenance medium solidified with agarose for short periods of time.

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*Correspondence to:* P. Vain

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*Particle Bombardment:* Plasmid DNA was precipitated on tungsten particles (M10, Sylvania) by mixing 10 µl of tungsten (1 mg/10 µl), 10 µl of DNA (1 µg/µl), 25 µl of 2.5 M $CaCl_2$, and 10 µl of 100 mM spermidine (free base). After 5 min at 4°C, 45 µl of the supernatant was removed and discarded. Bombardments were performed using the Particle Inflow Gun (Finer *et al.* 1992) with a helium pressure of 70 PSI and the solenoid set at 50 ms. Embryogenic maize cells were covered with a 500 µm baffle and placed at a distance of 17 cm from the filter unit containing the particles.

*Osmotic Treatments:* The influence of osmotic treatments on transient expression and stable transformation was tested by incorporating various concentrations of sorbitol and/or mannitol in the solidified MS medium used for the pre- and post-bombardment storage of the cells (Table 1). The initial osmotic treatment consisted of a 4 h pre-treatment with a 16 h post-treatment. The plasmid pGB5 (CaMV35S promoter:*Sh-1* intron:GUS coding region:NOS terminator) (Finer *et al.* 1992) was used for transient expression. Cultures were assayed for GUS activity (Jefferson 1987) 48 h after bombardment and the number of blue foci were counted. The plasmid pBARGUS (CaMV35S promoter:*Adh-1* intron:BAR coding region:NOS terminator + *Adh-1* promoter:*Adh-1* intron:GUS coding region:NOS terminator; Fromm *et al.* 1990) was used for stable transformation experiments.

*Post-Bombardment Treatments:* Selection for PPT-resistant maize lines was initiated 48 h after bombardment by placing the filter carrying the cells on a solidified MS medium containing 3-5 mg/l of bialaphos or glufosinate. Filters were transferred to fresh herbicide-containing medium every 15 d and resistant clones were isolated after 8 weeks. Plants were regenerated following the procedure of Green *et al.* (1983) and transferred to the greenhouse.

*Southern Hybridization Analysis:* DNA from calli and plants was isolated by the CTAB procedure (Saghai-Marnof *et al.* 1984). DNA was digested with *Kpn*I (which cleaves pBARGUS once), electrophoresed on a 0.8% agarose gel and transferred to Zetaprobe membranes (BioRad, Richmond, CA) using the protocol of Kempter *et al.* (1991). The CaMV 35S promotor was isolated as a *HindIII/BamHI* fragment from pUCGUS (Finer and McMullen 1990), random-prime labelled (Feinberg and Volgelstein 1983) and hybridized to membranes as previously described (Finer and McMullen 1991).

## Results and Discussion

*Influence of Osmotic Treatment on Transient Expression:*

Osmotic treatment of embryogenic maize cells for 4 h before and 16 h after bombardment enhanced transient expression of the GUS gene 2.7-fold (Fig. 1; Table 1). The osmoticum that was initially used consisted of a mixture of equimolar mannitol and sorbitol which was reported as the best osmoticum treatment for transformation of microorganisms (Armaleo *et al.* 1990; Shark *et al.* 1991). An average of approximately 9,000 blue foci were obtained from 100 µl PCV of cells placed on a medium containing 0.4 M osmoticum (Table 1). To determine the optimum osmotic treatment for transient expression, we tested media containing equimolar mannitol and sorbitol to give a final concentration of 0, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.8 M. Embryogenic maize cells placed on a medium containing from 0.4 to 0.6 M osmoticum gave the highest number of blue foci 2 d following bombardment (Fig. 2). For transient expression studies, the 0.4 M mannitol/sorbitol mixture was equivalent to use of 0.4 M mannitol (8,573 blue foci per bombardment) or 0.4 M sorbitol (8,256 blue foci) alone. The number of blue foci obtained per unit of PCV in this report represents a 6- to 7-fold improvement over the number of transient expression foci previously reported for maize (Gordon-Kamm *et al.* 1991).

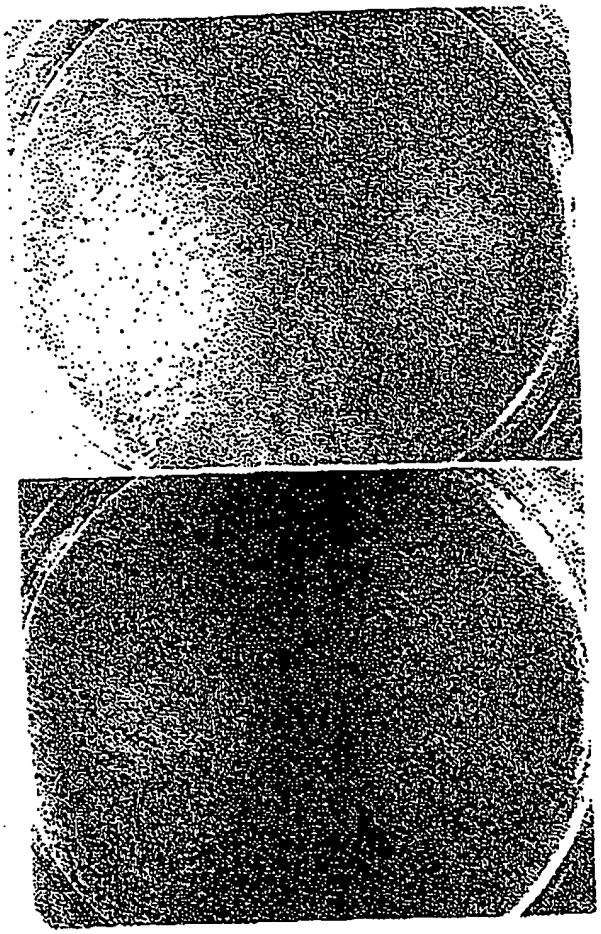

Figure 1. GUS expression in embryogenic maize cells 2 d after bombardment: a) without osmotic treatment b) with osmotic treatment (0.4 M osmoticum).

Further investigation of osmotic enhancement revealed a synergism between the pre- and post-osmotic treatment (Table 1). Pretreatment alone resulted in a 43% increase in transient expression while a post-treatment by itself had no effect. When a pre-treatment was performed with a post-treatment of various durations, the length of the post-treatment did not affect transient expression. A benefit from the post-treatment occurred only if the pre-treatment did not exceed 24 h (Table 1). With a 48 h pretreatment, the cells may have been altered (less responsive to transformation) from extended exposure to osmoticum-containing medium. This alteration could be osmotic adjustment (Turner and Jones 1980) or reduction of cell proliferation (growth rate; Hands *et al.* 1983) on an osmoticum-containing medium. It is interesting that a 48 h osmoticum post-treatment was not detrimental to transient expression, indicating that the cells were more sensitive to pre-bombardment manipulations. This sensitivity relates to transformation competency rather than sensitivity of the cells *per se*.

Table 1. Effect of osmotic treatments on transient GUS expression in maize cells after particle bombardment.

| Osmotic treatment (hours)[1] Before bombardment | After bombardment | # of blue foci for 100 µl PCV of cells |
|---|---|---|
| 0 | 0 | 3274 a[2] |
| 0 | 16 | 2608 a |
| 4 | 0 | 4691 b |
| 4 | 16 | 8789 c |
| 4 | 1 | 9376 a |
| 4 | 16 | 10178 a |
| 4 | 24 | 8283 a |
| 4 | 48 | 8077 a |
| 48 | 16 | 2908 a |
| 24 | 16 | 5676 b |
| 7 | 16 | 7236 bc |
| 4 | 16 | 8236 c |

[1]Osmotic treatment consisted of cell storage on an MS medium containing 0.4 M osmoticum.
[2]Entries followed by different letters are significantly different at P=0.05 by one way analysis of variance. Each value is the mean of 5-24 replications.

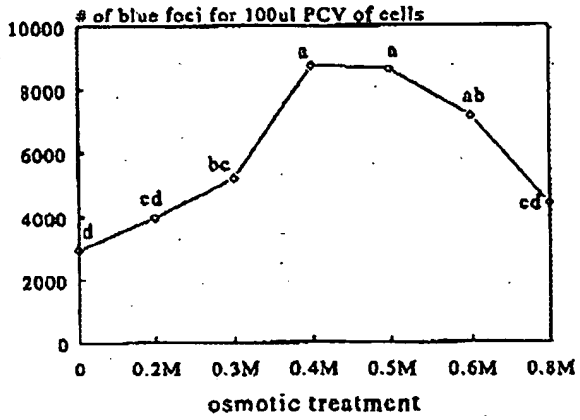


Figure 2. Effect of various concentrations of osmoticum on transient GUS expression in embryogenic maize cells. Equimolar mannitol and sorbitol were used to give the final molar concentration. A 4 h pretreatment was used with a 16 h post-treatment. [abcd]Entries followed by different letters are significantly different at P=0.05 by one way analysis of variance. Each value is the mean of 6 replications.

*Influence of Osmotic Treatment on Stable Transformation*

Bialaphos- and glufosinate-resistant clones were isolated 6 to 8 weeks following bombardment. Most of the herbicide-resistant lines exhibited intense GUS staining. Regardless of the level of GUS expression, all herbicide-resistant clones analyzed to date contained the introduced DNA(s) (Fig. 3). Although most of the resistant clones displayed a typical type II embryogenic callus phenotype, some of the callus lines underwent limited differentiation on the maintenance medium and developing embryos could be seen along the surface of the callus.

Maize cells placed on a medium containing 0.4 M osmoticum for 4 h before and 16 h after bombardment gave a 6.8-fold increase in the number of stable transformants obtained from 100 µl PCV of tissue (Table 2). From each 8,789 GUS-positive foci, 3.4 stably transformed embryogenic maize clones were recovered resulting in a transient-to-stable conversion frequency of 0.04%. Transient-to-stable conversion frequencies from less than 1% for embryogenic cells (Finer and McMullen 1990; Gordon-Kamm *et al.* 1990) to 10% (Russell *et al.* 1992; Spencer *et al.* 1990) for nonembryogenic cells have been reported. Although the transient-to-stable conversion frequency reported here is lower than others have reported for maize, the number of stably-transformed clones obtained per gram fresh weight of target tissue is 10-fold higher than previously reported (Gordon-Kamm *et al.* 1990).

In an attempt to optimize osmotic treatment effects for stable transformation of embryogenic maize cells, we tested various concentrations of osmoticum (0, 0.2, 0.3, 0.4, 0.5 final total molar concentration) as pre- and post-treatments. Only the 0.4 M treatment gave a significant (P=0.05 by one way analysis of variance) increase in stable transformation although all treatments resulted in an increase in the number of stable transformants versus the control (data not shown).

Table 2. Effect of osmotic treatment on stable transformation of maize using particle bombardment.

| Treatment | # of filters bombarded (100 μl PCV of cells per filter) | # of transformed clones[1] per filter per bombardment |
|---|---|---|
| Control | 32 | 0.5 [a2] |
| 0.4 M osmoticum | 14 | 3.4 [b] |

[1]Clones were determined to be transgenic via either GUS staining and/or Southern hybridization analyses.
[2]Entries followed by different letters are significantly different at P=0.05 according to Chi-square analysis.

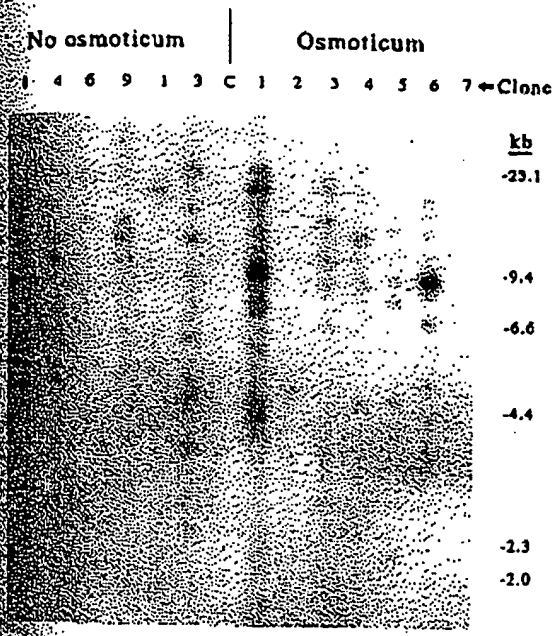


Figure 3. Southern hybridization analysis of glufosinate- and bialaphos-resistant clones obtained with or without osmoticum treatment. DNAs from nontransformed maize cultures (C) and glufosinate- or bialaphos-resistant clones (numbers refer to specific independent clones) was digested with *Kpn*I. The membrane was hybridized with the CaMV 35S promoter.

We believe that osmotic enhancement of transient expression and stable transformation of maize was facilitated through plasmolysis of the target cells. Plasmolyzed cells may be less likely to extrude their protoplasm following penetration of the cell by particles (Vasil, Castillo, Fromm and Vasil 1992; Vasil, Castillo, Malluleo *et al.* 1990; Sanford *et al.* 1992). The plasmolyzed state must be maintained for a few hours before and after bombardment to be the most effective. The filter paper that was used to support the cells may have buffered the cells from media changes so that the effects of the "osmotic pretreatment alone" may have been extended into the post-treatment period. In addition to direct exposure of cells to an osmotic agent, osmotic conditioning can also be attained by partial drying of the target tissue (Finer and McMullen 1990; Finer and McMullen 1991). The rationale behind partial drying was not discussed in these previous reports.

Southern hybridization analysis of clones obtained with or without osmoticum treatment revealed no clear differences in DNA integration patterns (Fig. 3). We anticipated possible differences in hybridizations patterns, specifically copy number of introduced DNA because plasmolyzed cells should be able to tolerate penetration by a larger number of particles, carrying more DNA into the cells. The multiple hybridizing bands represent DNA rearrangements either before or after integration, fragmented plasmids or plant-plasmid DNA borders.

In this report, the 2.7-fold enhancement in transient expression led to a 6.8-fold increase in stable transformation frequency. In addition to maintaining protoplasm integrity, the osmotic treatment may also have been beneficial for selection by reducing the cell growth and therefore improving selection efficacy.

*Transgenic Plant Recovery:*

Plants were routinely regenerated from transgenic embryogenic material (Fig. 4). Southern hybridization analysis of DNA from regenerated plants confirmed the presence of foreign DNA in regenerated plants (data not shown). Nontransformed plants were also obtained indicating the chimeric nature of some callus lines. The production of nontransformed plants could possibly be

Figure 4. Regenerated transgenic maize plants.

eliminated if the selective agent was maintained during the regeneration process (Fromm *et al.* 1990). Transgenic, GUS-positive progeny have been obtained from plants regenerated from callus lines containing the bar gene as well as the hygromycin resistance gene (data not shown).

## Conclusion

Use of the Particle Inflow Gun (PIG) with the proper cell conditioning/preparation has provided an efficient system for transformation of maize. For efficient transformation of plant cells using particle gun technology, both physical and biological parameters need to be evaluated. Improvement of the quality of the starting material as well as a reduction of stresses occurring during bombardment can provide major enhancements for plant transformation. This is the first report showing osmotic enhancement of transformation of embryogenic cells.

## Acknowledgments

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